MORAXELLA BOVIS CYTOTOXIN, CYTOTOXIN GENE, ANTIBODIES AND VACCINES FOR PREVENTION AND TREATMENT OF *MORAXELLA BOVIS* INFECTIONS

BACKGROUND OF THE INVENTION

Field of the Invention

The current invention concerns *Moraxella bovis* cytotoxin and a gene encoding *Moraxella bovis* cytotoxin. In particular, the invention concerns identification, isolation, cloning and identification of nucleotide sequence of the *Moraxella bovis* gene *mbxA* and adjacent genes *mbxB*, *mbxC* and *mbxD*, partial purification of the native cytotoxin, preparation of a purified native and recombinant *Moraxella bovis* cytotoxin, an amino acid sequence of the cytotoxins, preparation of antibodies against the *Moraxella bovis* cytotoxin, preparation of vaccines against *Moraxella bovis* and a method for prevention and treatment of infectious bovine keratoconjunctivitis caused by *Moraxella bovis*.

Background and Related Disclosures

Infectious bovine keratoconjunctivitis (IBK) also called pinkeye, caused by *Moraxella bovis (M. bovis)* infection ranks as one of the most important cattle diseases in the United States and abroad. Annual estimated incidence of the disease is 5% of all beef cattle with greater than 50% of all herds affected. Epizootics also occur with case attack rates approaching 90-100% of yearling cattle. Affected cattle do not eat and fail to gain weight. Economic losses due to lower market weights of affected cattle and calves and ocular scarring and treatment associated expenses are estimated to

exceed $150,000,000 annually. Effective control measures that could substantially reduce this expense are not available as the commercially available vaccines have low efficacy.

Animals affected with IBK exhibit corneal ulceration, edema, ocular pain, photophobia and lacrimation. Control of IBK using antimicrobial treatments has been only partially successful. Antimicrobials have been used to eliminate the carrier state in experimental and field trials, but these treatments have drawbacks that include emergence of resistant bacteria in cattle and the general environment, potential for adulteration of the nation's food supply, high cost and marginal economic benefit.

Topical and oral administration of antibiotics has proven somewhat effective for treatment of pinkeye, but the high cost and losses associated with the ongoing disease often outweigh the benefits of such treatment. Topical therapy alone does not eliminate *M. bovis* from non-ocular sites such as nasal choanae and vagina where it is known to reside.

Consequently, an effective vaccine or antibody for prevention and treatment of IBK would be an important component of a successful IBK control program.

So far, vaccines prepared from killed or live bacteria, isolated pili, or ribosomes have had limited benefit for immunization of cattle in the field (Proc. Am. Assoc. Bov. Pract., 20:26-32 (1987)). Nevertheless, a large body of field and experimental data exist to show that acquired resistance to pinkeye does develop after the natural occurrence of the infection.

At least two attributes, namely pili and cytotoxin (leukotoxin/hemolysin/cytolysin), of the *M. bovis* organism are important in the pathogenesis of IBK. *M. bovis* pili

facilitate adhesion of the bacterium to the corneal epithelium and are required for colonization. Pili are highly immunogenic, but have antigenic diversity due to the presence of at least two structural pilin genes and variability in the amino acid composition of the pilin molecule. Variability in pilus gene expression is regulated by a site-specific DNA inversion system. Limited antigenic cross-reactivity was found in vaccines prepared from heterologous pili. *M. bovis* pili in monovalent or multivalent formulations have demonstrated variable efficacy, attributed to pilus gene inversion that occurs during infection (Vet. Microbiol., 45:129-138 (1995)). The emergence of novel pili serotypes during epizootics of IBK has been documented (Am. J. Vet. Res., 50:1437-1441 (1989)). These studies suggest that because of the pili heterogeneity pilus based vaccines will not be 100% effective at preventing IBK.

The *Moraxella bovis* cytotoxin (hemolysin) is an important component of immunity to IBK (FEMS Microbiol. Lett., 124:69-74 (1994)).

Moraxella bovis produces a heat labile, approximately 100 kD cytotoxin (hemolysin) that causes cell lysis by forming pores in cell membranes via a calcium-dependent process (Infect. Immunol., 59:1148-1152 (1991)). *In vitro*, culture filtrates from broth cultures of hemolytic but not nonhemolytic strains of *M. bovis* cause lysis of bovine neutrophils (Am. J. Vet. Res., 51:191-196 (1990)). *In vivo*, the ocular damage caused by a purified hemolytic and cytotoxic fraction of *M. bovis* mimics the lesions seen in naturally occurring IBK (Vet. Microbiol., 42:15-33 (1994)). Unlike the pilin molecule, the *M. bovis* cytotoxin appears to be more

conserved between different *M. bovis* isolates. Serologic studies show that antihemolytic antibodies recognize hemolysin from different strains of M. bovis (Am. J. Vet. Res., 46:1011-1014 (1985)) and, thus, form an important component of immunity to IBK (FEMS Microbiol. Lett., 124:69-74 (1994)).

In view of the severity of consequences of infectious bovine keratoconjunctivitis, it would be important to have available an effective vaccine and/or anti-*Moraxella bovis* antibodies that could prevent this condition.

This invention, therefore, concerns an effective IBK vaccine, anti-*Moraxella bovis* antibodies and to that end an isolation and partial purification of the native *Moraxella bovis* cytotoxin, a characterization of the *M. bovis* cytotoxin gene, identification of *M. bovis* cytotoxin amino acid sequence, preparation of recombinant *M. bovis* cytotoxin and determination of its efficacy in a vaccine for prophylaxis and treatment of IBK.

All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety.

SUMMARY

One aspect of the current invention is a vaccine for prevention of infectious bovine keratoconjunctivitis comprising a purified native or recombinant *Moraxella bovis* cytotoxin.

Still another aspect of the current invention is a DNA sequence of the *Moraxella bovis* gene depicted by SEQ ID NO: 1.

Still another aspect of the current invention is a DNA sequence of the *Moraxella bovis* gene B depicted by SEQ ID NO: 30.

Still another aspect of the current invention is a DNA sequence of the *Moraxella bovis* gene C depicted by SEQ ID NO: 31.

Still another aspect of the current invention is a DNA sequence of the *Moraxella bovis* gene D depicted by SEQ ID NO: 36.

Yet another aspect of the current invention is an amino acid sequence of *Moraxella bovis* cytotoxin A depicted by SEQ ID NO: 2 or a partially purified native or recombinantly prepared cytotoxin comprising SEQ ID NO: 2.

Yet another aspect of the current invention is an amino acid sequence of *Moraxella bovis* cytotoxin B depicted by SEQ ID NO: 30 or a partially purified native or recombinantly prepared cytotoxin comprising SEQ ID NO: 18.

Yet another aspect of the current invention is an amino acid sequence of *Moraxella bovis* cytotoxin C depicted by SEQ ID NO: 31 or a partially purified native or recombinantly prepared cytotoxin comprising SEQ ID NO: 32.

Yet another aspect of the current invention is an amino acid sequence of *Moraxella bovis* cytotoxin D depicted by SEQ ID NO: 36 or a partially purified native or recombinantly prepared cytotoxin comprising SEQ ID NO: 37.

Still another aspect of the current invention is a method for purification or partial purification of the *Moraxella bovis* cytotoxin and its stabilization.

Still another aspect of the current invention is a method for prevention of IBK in cattle and calves by vaccinating said cattle or calves with a vaccine comprising a partially purified native or recombinant *Moraxella bovis* cytotoxin comprising SEQ ID NO: 2.

BRIEF DESCRIPTION OF FIGURES

Figure 1 is a graph illustrating neutralization of *M. bovis* leukotoxin in bacterial filter permeates by anti T+ serum.

Figure 2 is an autoradiogram of the pooled and concentrated void volume fraction of diafiltered retentate from T+ and T- cultures chromatographed on a Superose 6HR column.

Figure 3 shows nucleotide (SEQ ID NO: 1) and deduced amino acid (SEQ ID NO: 2) sequences of M. bovis RTXA (MbxA) gene.

Figure 4 shows the alignment of the deduced amino acid sequence of M. bovis RTXA (MbxA) gene (SEQ ID NO: 2) and RTX toxins of *M. (Pasteurella) haemolytica* (LktA) (SEQ ID NO: 3), *A. pleuropneumoniae* (ApxIA) (SEQ ID NO: 4) and *E. coli* (HlyA) (SEQ ID NO: 5).

Figure 5 is an autoradiogram demonstrating MbxA presence in culture supernatants of T+, and Tifton I and absence in T- culture supernatants.

Figure 6 is an autoradiogram of culture supernatants from a Tifton I broth culture at 3, 4, 4.5, 5 and 6 hours time points.

Figure 7 shows the percent neutralization of cytolysis and hemolysis by non-immune and immune sera alone or preabsorbed with a recombinant expressed carboxy terminus of MbxA.

Figure 8 shows nucleotide (SEQ ID NO: 30) and deduced amino acid (SEQ ID NO: 18) sequences of M. bovis RTXB (MbxB) gene.

Figure 9 shows the alignment of the deduced amino acid sequence of M. bovis RTXB (MbxB) gene (SEQ ID NO: 18) and RTX toxins from *M. (Pasteurella) haemolytica* (LktB) (SEQ ID NO: 19), *A. pleuropneumoniae* (ApxIIB) (SEQ ID NO:20) and *E. coli* (HlyB) (SEQ ID NO: 21).

Figure 10 shows nucleotide (SEQ ID NO: 31) and deduced amino acid (SEQ ID Nos: 32) sequences of M. bovis RTXC (MbxC) gene.

Figure 11 shows the alignment of the deduced amino acid sequence (SEQ ID NO: 32) of M. bovis RTXC (MbxC) gene and RTX toxins of *M. (Pasteurella) haemolytica* (LktC) (SEQ ID NO: 33), *A. pleuropneumoniae* (ApxIIC) (SEQ ID NO:34) and *E. coli* (HlyC) (SEQ ID NO: 35).

Figure 12 shows nucleotide (SEQ ID NO: 36) and deduced amino acid (SEQ ID NO: 37) sequences of M. bovis RTXD (MbxD) gene.

Figure 13 shows the alignment of the deduced amino acid sequence (SEQ ID NO: 37) of M. bovis RTXD (MbxD) gene and RTX toxins of *M. (Pasteurella) haemolytica* (LktD) gene (SEQ ID NO: 38), *A. pleuropneumoniae* (ApxIID) gene (SEQ ID NO: 39) and *E. coli* (HlyD) gene(SEQ ID NO: 40).

Figure 14 is a graph showing the cumulative number of calves with ulcers in one eye vaccinated with *M. bovis* cytotoxin, adjuvant alone, or saline (control) over time.

Figure 15 is a graph showing a number of calves affected weekly in one group of vaccinated calves and in controls.

Figure 16 is a graph showing cumulative proportion of ulcerated calves during the trial with recombinant cytotoxin vaccine, saline alone or adjuvant alone.

DEFINITIONS

As used herein:

"Cytotoxin," "cytolysin" or "hemolysin" means a protein produced by *Moraxella bovis* that is lytic for cells. The cytotoxin gene encodes 927 amino acids depicted by SEQ ID NO: 2 and is homologous to the internal regions of the published repeats in the structural toxin (RTX) toxins of *E. coli*, *M. (Pasteurella) haemolytica* and *Actinobacillus* species.

"Antigen" or "*M. bovis* antigen" means *M. bovis* cytotoxin as defined above.

"Functionality" or "functional characteristics" means the interaction of antibodies against *M. bovis* cytotoxin, such that the antibody inhibits the activity of *M. bovis* cytotoxin and retards/prevents development of IBK.

"ISCOM" means a partially purified cytotoxin formulated in an immunostimulating complex comprising *Quillaja saponins (Quil A)*, cholesterol and phospholipids.

"IBK" means infectious bovine keratoconjunctivitis (pinkeye) caused by *Moraxella bovis*.

"*M. bovis*" or "*Moraxella bovis*" means the etiologic agent of IBK which expresses a cytotoxin that lyses corneal epithelial cells and neutrophils and results in corneal ulceration in cattle and calves. Pathogenic isolates of *M. bovis* are hemolytic; non-pathogenic isolates are nonhemolytic.

"*mbxA*" means a gene encoding *M. bovis* cytotoxin, said gene comprising a nucleotide sequence of 2784 base pairs depicted as SEQ ID NO: 1.

"MbxA" means a protein encoded by *mbxA* gene described as SEQ ID NO: 2.

"Leukotoxic" activity means biological activity which causes lysis of bovine neutrophils and lymphoma cells.

"Hemolytic" activity means biological activity which causes lysis of red blood cells.

"Corneotoxic" activity means biological activity which causes lysis of corneal epithelial cells.

"KDO" means 2-keto-3-deoxyoctonate.

"Antibodies" means proteins which structurally interact with the target antigen, i.e., *M. bovis* cytotoxin, and are produced when the antigen is introduced into an animal, such that they stimulate the immune system. The term also includes antibodies produced *in vitro*, such as recombinant antibodies, antibodies produced by hybridoma cell cultures and chimeric proteins, as well as hybridoma cells and chimeric constructs introduced into the host to provide an *in vivo* antibody.

"Antibodies to *M. bovis* antigen" means proteins which structurally interact with the target antigen and inhibit or detect infectious bovine keratoconjunctivitis.

"Monoclonal antibodies" means the monovalent antibodies produced by B cells fused to immortalized cells producing specific antibody to *M. bovis* antigen.

"Polyclonal antibodies" means antibodies directed at *M. bovis* antigen which are not monovalent and are the products of multiple B cells in character.

"*M. bovis* antigen DNA" means the sequence of about 2784 nucleotides identified as SEQ ID NO: 1 which encodes a protein comprising an amino acid sequence depicted by SEQ ID NO: 2.

"Vaccine" means a protein, recombinant protein, DNA, RNA, or a fragment thereof preserved from *M. bovis* cytotoxin which, upon administration to a host, is able to provoke an immune

response including but not limited to the production of antibodies, cytokines and other cellular responses.

"Prevention or prophylaxis" means the passive or active immunization with antibodies or vaccines of the invention such that disease or infection does not occur.

"Treatment" means therapeutic use of any protein or antibody to inhibit existing infection in a cattle or calf host.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates generally to infectious bovine keratoconjunctivitis, to vaccines, antibodies, purified or partially purified native or recombinant proteins, DNAs, RNAs and methods for prophylaxis and treatment of infectious bovine keratoconjunctivitis (IBK).

More specifically, the invention concerns identification, isolation, and purification or partial purification of native *M. bovis* antigen (cytotoxin) which causes IBK in cattle and calves, determination of its biological activity, identification and isolation of an *M. bovis* genes *mbxA, mbxB, mbxC and mbxD,* and determination of their nucleotide sequences, deducing their amino acid sequences based on the DNA sequences, partial sequencing of native partially purified cytotoxin, cloning of *M. bovis* genes *mbxA, mbxB, mbxC* and *MbxD,* expression of recombinant proteins, identification of the amino acid sequences of the expressed proteins and their purification, and preparation of *M. bovis* cytotoxin containing vaccines or polyclonal or monoclonal antibodies against such cytotoxin.

I. Native *Moraxella bovis* Cytotoxin

Moraxella bovis is a bacteria isolated from a cattle infected with *Moraxella bovis* and suffering from infectious bovine keratoconjunctivitis. *M. bovis* strains are pathogenic or nonpathogenic and different isolates may be found in different regions.

A. *M. bovis* Strains and Isolates

A cytolytic strain *M. bovis* (T+) and its noncytolytic subculture (T-), as well as cytolytic strains EPP63 and GLN63 were obtained from Dr. G. W. Pugh, Ames, Iowa. Additional isolates Tifton 1, Tifton 2, GA3, LS-2, LS-3, LS-4, LS-5, LS-6, LS-7 and LS-8 were obtained from field specimens submitted for determination of IBK infections from various states. The strains were isolated according to Example 1.

B. Characteristics of Native *M. bovis* Cytotoxin

Moraxella bovis produces a toxin, herein called cytotoxin, which causes cell lysis.

The cytotoxin of *Moraxella bovis* (*M. bovis*) is a transmembrane pore forming molecule that shares antigenic homology to the *E. coli* α-hemolysin, and is excreted in particulate complexes with the proteins during log phase growth. The cytotoxin has leukotoxic, corneotoxic, and hemolytic activities. Once released into the media, the liberated cytotoxin and protein aggregates can be separated from the whole bacteria by filtration through polycarbonate membranes of a mean pore diameter 0.22 μM, and can be retained by filters with molecular weight cut-offs that are >100 kDa.

Cytotoxin is an unstable molecule with activity dissipating rapidly after removal from the bacterial cell. Its pathogenic activity is irreversibly inactivated by a number of chemical, enzymatic, and physical treatments,

including trypsin, sodium dodecyl sulfate, high incubation temperature (>37°C), and calcium chelation.

The molecular complexity and the lability of the cell free cytotoxin has precluded purification to a monomolecular state.

C. Immunogenicity of *M. bovis* Strains

To investigate the immunogenic potential of cytotoxin, characterization of cytotoxin of *M. bovis* strains from diverse regions of the United States were undertaken.

For this purpose, leukotoxins were obtained from nine field strains and three isolates. These leukotoxins were submitted to neutralization assay as described in Example 21 with immune serum or pre-immune serum. Results are seen in Figure 1.

Figure 1 illustrates neutralization of *M. bovis* leukotoxin in bacterial filter permeates from isolates Tifton 1, Tifton 2, LS-2, LS-3, LS-4, LS-5, LS-6, LS-7, LS-8 and in three cytolytic strains T+, EPP63 and GLN63 with immune rabbit anti-T+ serum compared to neutralization of these permeates with pre-immune serum from the same rabbit. The pre-immune serum was used as the negative control. In this study, the same bacterial filter permeates were incubated with either the immune serum or pre-immune serum under the same conditions. The leukotoxic activity was measured by LDH assay.

As seen in Figure 1, the undiluted anti-T+ diafiltered retentate serum inhibited the cytotoxic activity of all strains and isolates. No such inhibition was observed in samples incubated with pre-immune serum.

These results clearly confirm that the leukotoxic activity of cytotoxin is shared by *M. bovis* strains and

isolates and suggest that a vaccine containing such cytotoxin would immunize the cattle against IBK.

II. Cytotoxin Gene, Protein, Recombinant Protein and DNA

Following the discovery and confirmation that the cytotoxin protein is an antigen which could be used as a vaccine for cross-strain protection against IBK, cloning, characterization and sequencing of the *M. bovis* cytotoxin genes, expression of the proteins encoded by these genes and the amino acid sequencing of HPLC purified peptides derived from native cytotoxin was undertaken.

A. Isolation of *M. bovis* Cytotoxin for Amino Acid Sequence Analysis

For amino acid sequence analysis, the *M. bovis* cytotoxin/cytotoxin peptides were partially purified by the procedure described in Example 14. To identify proteins that were unique to the hemolytic strain T+, purified culture extracts from hemolytic T+ and nonhemolytic T- *M. bovis* strains were compared by Western blotting using the procedure described in Example 15.

Several candidate proteins for the *M. bovis* cytotoxin with molecular masses ranging from 55 to 75 kDa were identified as shown in Figure 2.

Figure 2 is the autoradiogram of the pooled and concentrated void volume fraction of diafiltered retentate from T+ and T- cultures chromatographed on a Superose 6 column. The rabbit antiserum to column purified cytotoxin, in 1:400 dilution, was used as the primary antibody. Molecular mass markers 47, 84, 116 and 208 kDa are indicated. Protein bands in T+ lane ranging from 55 to 75 kDa were found to represent *M. bovis* cytotoxin protein/peptides that were unique

to T+ diafiltered retentate. A 70 kDa protein in Superose 6 chromatographed T+ diafiltered retentate was subsequently identified on a Coomassie blue stained SDS polyacrylamide gel and was the source for two peptides designated peptides #23 and #26. These peptides were sequenced by N-terminal Edman degradation chemistry.

The respective amino acid sequences for two tryptic peptides #23 and #26 that were derived from the 70 kDa band were FLSELNKELEAE (SEQ ID NO: 6) and FNDIFHSGEGDDLLDSGA (SEQ ID NO: 13).

BLAST database searches identified relatedness between the two peptides and the deduced amino acid sequences from RTX A genes of *E. coli, M. haemolytica, A. suis, A. pleuropneumoniae,* and *A. actinomycetemcomitans*. These alignments also predicted that peptide #23 was amino to peptide #26. Sequence and orientation-specific degenerate PCR primers 23A and 26A designed from reverse translating the amino acid sequences for peptides #23 and #26, respectively, amplified an approximately 850 bp fragment of genomic DNA from *M. bovis* Tifton I strain.

The deduced amino acid encoded by this region exhibited homology to the RTX family of bacterial exoproteins. Complete cloning and sequencing of *mbxA* was subsequently performed using a PCR based approach as described herein.

B. Homology with RTX Toxins

Amino acid sequencing of HPLC purified peptides derived from the purified cytotoxin protein has yielded amino acid sequences which show homology to internal regions of the published RTX toxins of *E. coli, M. haemolytica* and

Actinobacillus spp. Regions of homology are seen in Table 4 and Table 5.

Table 4

Amino Acid Alignment

M. bovis cytotoxin	F	L	S	E	L	N	K	E	L	E	A	E	SEQ ID NO: 6
M. haemolytica leukotoxin	F	L	L	N	L	N	K	E	L	Q	A	E	SEQ ID NO: 7
E. coli alpha hemolysin	I	L	S	Q	Y	N	K	E	Y	S	V	E	SEQ ID NO: 8
A. pleuropneumoniae hemolysin	F	L	I	N	L	N	K	E	L	Q	A	E	SEQ ID NO: 9
A. pleuropneumoniae hemolysin	L	L	S	Q	Y	N	K	E	Y	S	V	E	SEQ ID NO: 10
A. suis cytotoxin	F	L	I	N	L	N	K	E	L	Q	A	E	SEQ ID NO: 11
Consensus sequence	F	L	.	.	L/Y	N	K	E	L/Y	.	A/V	E	SEQ ID NO. 12

Table 4 shows amino acid sequence of *M. bovis* cytotoxin peptide #23 aligned with known published RTX toxin amino acid sequences.

Table 5

Alignment of Fragment #26

M. bovis cytotoxin	F	N	D	I	F	H	S	G	E	G	D	D	L	L	SEQ ID NO: 13
A. pleuropneumoniae serotype 8 cytolysin	F	R	D	I	F	H	G	A	D	G	D	D	L	L	SEQ ID NO: 14
A. actinomycetemcomitans leukotoxin	F	N	D	V	F	H	G	H	D	G	D	D	L	I	SEQ ID NO: 15
A. pleuropneumoniae serotype 2 cytolysin	F	R	D	I	F	H	G	A	D	G	D	D	L	L	SEQ ID NO: 16
Consensus sequence	F	.	D	.	F	H	G	A	D	G	D	D		L/i	SEQ ID NO: 17

Table 5 shows amino acid sequence of *M. bovis* cytotoxin peptide peak #26 aligned with known published amino acid sequences of RTX toxins.

Obtained results show that a relationship exists between the *M. bovis* cytotoxin and the RTX family of toxins. These results are further supported by the earlier findings of other investigators that a monoclonal antibody to the RTX toxin of *E. coli*, namely alpha-hemolysin, recognized a protein in hemolytic and cytolytic extracts from hemolytic but not nonhemolytic *M. bovis* (Vet. Microbiol., 43:183-196 (1995)).

C. Deduced Amino Acid Sequences

Amino acid sequences of products MbxA, MbxB, MbxC and MbxD of the four genes *mbxA*, *mbxB*, *mbxC* and *mbxD* were deduced. The peptide MbxA is the structural cytotoxin. Peptides MbxB, MbxC and MbxD are involved in *M. bovis* cytotoxin activity.

Specifically, MbxA peptide is encoded by the *mbxA* gene. DNA sequence of the *mbxA* gene was determined according to the Example 17. Plasmid DNA was isolated with Qiagen plasmid minipreps (Qiagen, Valencia, CA). The DNA sequence of the complete cytotoxin gene was obtained by sequencing PCR products directly or PCR products cloned into pCR2.1-TOPO (Invitrogen) with a TOPO TA cloning kit (Invitrogen). PCR primers C-down and B-up, corresponding to conserved regions within respective RTX C and B genes of other RTX toxin producing bacteria, were used for PCR amplification. These primers amplified a 4 kb region that contained an open reading frame (ORF) of 2784 nucleotide bases including stop codon depicted as sequence SEQ ID NO: 1, seen in Figure 3. This gene was designated *mbxA*.

The MbxA protein encoded by *mbxA* gene was deduced to be 927 amino acids with a predicted molecular mass of 98.8 kDa (SEQ ID NO: 2).

There was approximately 40-50% identity between deduced amino acid sequences of *mbxA* and other *RTXA* genes. Specifically, there was 50.4% identity with *M. haemolytica* LktA, 49.1% with *A. pleuropneumoniae* ApxIIA, 48.7% with *A. suis* ClyIIA, 43.5% with *E. coli* HlyA, and 40.9% with *A. actinomycetemcomitans* LtA. A sequence alignment of MbxA with LktA, ApxIIA, and HlyA is shown in Figure 4.

Figure 4 shows alignment of the deduced amino acid sequences of MbxA and RTX toxins of *M. (Pasteurella) haemolytica* (LktA), *A. pleuropneumoniae (ApxIIA)* and *E.coli* (HlyA). Black boxes indicate identical amino acids. Gray boxes indicate highly similar amino acids.

The location of peptides #23 and #26 derived from trypsin digestion of the 70 kDa protein are indicated except that the residue at position 720 was designated serine by N-terminal Edman degradation chemistry. The sequences corresponding to 6 glycine rich repeats are indicated on the top MbxA sequence by double lines. The expressed recombinant internal peptide corresponded to amino acid 438 through 713. The expressed recombinant carboxy peptide corresponded to amino acids 643 through 927. Lysines at positions 536 and 660 (marked by *) are hypothetical acylation modification sites as determined by relatedness of the neighboring amino acids to published consensus sequences (Infect. Immun., 64:3081-7 (1996) and Microbiol. Mol. Biol. Rev., 62:309-33 (1998)) surrounding modified lysine residues of HlyA.

MbxA protein has been found to have as much as 50% identity with other RTX toxins and this homology extends over the length of the protein. The carboxy terminus of MbxA features glycine rich repeats that are characteristic of RTX proteins. Specifically, there are six glycine-rich repeats in the carboxy terminal 325 amino acids of MbxA. Four of these exactly match the predicted L/V-X-G-G-X-G-N/D-D-X (SEQ ID NO: 29) consensus for glycine repeats in RTX toxins.

Identification of MbxA protein in *M. bovis* in culture supernatants is illustrated in Figure 5.

Figure 5 is an autoradiogram demonstrating cytotoxin MbxA in culture supernatants of T+ and Tifton I. Three hour culture supernatants from T+, T- and Tifton I (Tif I) were electrophoresed on a 3.9%/7.5% SDS-polyacrylamide gel, and transblotted to Immobilon-P (Millipore) and probed with rabbit antisera against the expressed recombinant carboxy peptide (1:250 dilution). Molecular mass markers 45 kDa, 66 kDa, 97 kDa, 116 kDa and 200 kDa are indicated. Two proteins with molecular masses of 102 and 105 kDa were present in culture supernatants from both T+ and Tifton I. No immunoreactive proteins were present in the T- culture supernatant. These proteins were concluded to represent full length MbxA protein.

During longer incubations (4-6 hours), smaller immunoreactive proteins with molecular masses ranging from 52 to 91 kDa were identified in the culture supernatant as shown in Figure 6.

Figure 6 is an autoradiogram of culture supernatants from a Tifton I broth culture at 3, 4, 4.5, 5 and 6 hour time points. The blot was probed with rabbit antiserum against the expressed recombinant internal peptide encoded by *MbxA* gene

(amino acids 438 through 713). Molecular mass markers 45, 66, 97, 116 and 200 kDa are indicated.

The appearance of proteins that were smaller than the 102 and 105 kDa predicted full length cytotoxin, following 4-6 hours of incubation, as seen in Figure 6, has been attributed to proteolysis. Hemolytic *M. bovis* produces numerous hydrolytic enzymes including C4 esterase, C8 esterase-lipase, C14 lipase, phosphoamidase, phosphatase, leucine and valine aminopeptidases and gelatinase. Proteolysis is believed to account for the difference in mass between the 70 kDa protein selected for amino acid sequencing and the 98.8 kDa molecular mass predicted for full length MbxA. The data indicate that *M. bovis* could also express smaller molecular mass proteins that share epitopes with the internal region MbxA.

To confirm that MbxA encoded the *M. bovis* cytotoxin, neutralization assays were performed with antisera that was preabsorbed with the recombinant expressed carboxy terminus of MbxA (carboxy peptide). Results are seen in Figure 7.

Figure 7 shows percent neutralization (± 1 standard deviation) of cytolysis (top) and hemolysis (bottom) by non-immune sera alone or preabsorbed with the carboxy peptide. Cytotoxin was tested for neutralization with nonimmune fetal bovine serum (FBS); with preimmune sera A and B obtained from two rabbits A and B; with immune rabbit anti T+ sera obtained from T+ rabbit polyclonal antisera to culture filtrates from *M. bovis* strain T+; and with immune sera A and B obtained from rabbits A and B vaccinated with the carboxy peptide.

As seen in Figure 7, rabbit anti T+ sera lost over 50% of its hemolytic and cytolytic neutralizing capacity following preabsorption with the carboxy peptide. Rabbit antisera

against the carboxy peptide neutralized the hemolytic and cytolytic activity of native *M. bovis* cytotoxin. The neutralizing capacity of the sera designated A and B obtained from rabbit A and rabbit B was nearly equivalent to, or higher than the neutralizing capacity of the rabbit anti T+ antisera. Similar to rabbit anti T+ antisera preabsorbed with the carboxy peptide, rabbit A and B sera preabsorbed with the carboxy peptide lost over 60% of lysis neutralizing capacity relative to the unabsorbed samples.

The high percent neutralization of cytolysis for rabbit B serum occurred because the serum exhibited less cytolysis than the cytolysis negative control (TBS $CaCl_2$ buffer). The percent neutralization of hemolysis was < 0 for fetal bovine serum because these samples exhibited more hemolysis than the hemolysis positive control (Tifton I diafiltered retentate + TBS $CaCl_2$ buffer). When incubated with indicator cells, the carboxy peptide alone was neither hemolytic nor cytolytic.

D. Cytotoxin Protein MbxA and other Proteins

Cytotoxin protein MbxA comprises 927 amino acids depicted as SEQ ID NO: 2. Amino acid SEQ ID NO: 2 comprises two peptides identified as peptide #23 and peptide #26.

Peptide #23 of MbxA cytotoxin is depicted by the amino acid sequence identified as SEQ ID NO: 6. The amino acid sequence of peptide #23 aligns with known published RTX amino acid sequences of *M. haemolytica leukotoxin* (SEQ ID NO: 7), *E. coli* alpha hemolysin (SEQ ID NO: 8), *A. pleuropneumoniae hemolysin* (SEQ ID NO: 9), *A. pleuropneumoniae hemolysin* (SEQ ID NO: 10), *A. suis cytotoxin* (SEQ ID NO: 11), as shown in Table 4 (supra), which also lists a consensus sequence identified as SEQ ID NO: 12.

Peptide #26 of MbxA cytotoxin is depicted by the amino acid sequence identified as SEQ ID NO: 13. The peptide #26 aligns with *A. pleuropneumoniae* serotype 8 cytolysin (SEQ ID NO: 14), *A. actinomycetemcomitans* leukotoxin (SEQ ID NO: 15) and *A. pleuropneumoniae* serotype 2 cytolysin (SEQ ID NO: 16), as seen in Table 5 (supra). Table 5 also lists a consensus sequence (SEQ ID NO: 17).

Additionally, the deduced amino acid sequences of products of *M. bovis* RTXB (*MbxB*), RTXC (*MbxC*) and RTXD (*MbxD*) genes were determined.

The deduced amino acid sequence of product of *M. bovis* RTXB gene(*MbxB*) is depicted by SEQ ID NO: 18. The MbxB protein encoded by *mbxB* gene was deduced to be 739 amino acids . Amino acid sequence of the MbxB protein is seen in Figure 8.

Deduced amino acid sequence MbxB aligned with other products of the RTXB genes, as seen in Figure 9. Figure 9 shows an alignment of *M. bovis* (MbxB) with the deduced amino acid sequences of RTXB genes from *M. (Pasteurella) haemolytica* (LktB) (SEQ ID NO: 19), *A. pleuropneumoniae* (ApxIB) (SEQ ID NO: 20) and *E. coli* (HlyB) (SEQ ID NO: 21). Black boxes in Figure 9 indicate identical amino acids. Gray boxes indicate highly similar amino acids.

The deduced amino acid sequence of product of *M. bovis* RTXC gene (*MbxC*) is depicted by SEQ ID NO: 32. The MbxC protein encoded by *mbxC* gene was deduced to be 169 amino acids. Amino acid sequence of the MbxC protein is seen in Figure 10.

Deduced amino acid sequence MbxC aligned with other products of the RTXC genes, as seen in Figure 10. Figure 10 shows an alignment of *M. bovis* (MbxC) with the deduced amino

acid sequences of RTXC genes from *M. (Pasteurella) haemolytica* (LktC) (SEQ ID NO: 33), *A. pleuropneumoniae* (ApxIC) (SEQ ID NO: 34) and *E. coli* (HlyC) (SEQ ID NO: 35), as seen in Figure 11. Black boxes in Figure 11 indicate identical amino acids. Gray boxes indicate highly similar amino acids.

The deduced amino acid sequence of MbxD protein encoded by the *M. bovis* RTXD gene (*MbxD*) is depicted by SEQ ID NO: 37. The MbxD protein encoded by *mbxD* gene was deduced to be 476 amino acids. Amino acid sequence of the MbxD protein is seen in Figure 12.

Deduced amino acid sequence MbxD aligned with other products of the RTXD genes is seen in Figure 13. Figure 13 shows an alignment of *M. bovis* (MbxD) with the deduced amino acid sequences of RTXD genes from *M. (Pasteurella) haemolytica* (LktB) (SEQ ID NO: 38), *A. pleuropneumoniae* (ApxIB) (SEQ ID NO: 39) and *E. coli* (HlyB) (SEQ ID NO: 40). Black boxes in Figure 13 indicate identical amino acids. Gray boxes indicate highly similar amino acids.

The above findings conclusively documents the presence of RTX gene (*mbxA*) and other RTX genes in *M. bovis*. The *mbxA* gene encodes a protein MbxA which has been found to be the etiologic agent of infectious bovine keratoconjunctivitis. The data also show that the protein expressed from this gene is responsible for the hemolytic and cytolytic activity present in culture extracts from pathogenic *M. bovis*, and thus proves that the *M. bovis* cytotoxin is an RTX toxin.

E. Cytotoxin Gene *mbxA* and *mbxB*, *mbxC* and *mbxD* Genes

Cytotoxin gene *mbxA* and othe RTX genes, namely *mbxB*, *mbxC* and *mbxD* belong to a family of RTX genes encoding bacterial pore-forming toxins. RTX genes are composed of four genes

organized 5'-C-A-B-D-3'. The *mbxC* gene is located upstream of *mbxA*. The *mbxB* and *mbxD* are located downstream of *mbxA*.

The product of the RTXA gene is the structural toxin which is activated by the RTXC gene product to become hemolytic. Such activation is mediated by fatty acylation of conserved lysines. The activated toxin is secreted by membrane transport proteins encoded by RTXB and RTXD genes and a third protein TolC.

The cytotoxin gene *mbxA*, disclosed herein, has similar properties to other RTX genes and, as described above, its encoded protein MbxA has 50% identity with the deduced amino acid sequence of *M. haemolytica* leukotoxin. MbxA protein has 6 glycine-rich repeats in the carboxy terminus and two putative lysine acylation sites which are necessary for toxin activation.

RTX toxin gene *mbxA* was now shown to encode the *M. bovis* cytotoxin.

1. Structures of the *mbxA*, *mbxB*, *mbxC* and *mbxD* Genes

DNA sequence identified as SEQ ID NO: 1 represents the *M. bovis* RTXA gene *mtxA*. The nucleotide sequence of the *mtxA* gene is shown in Figure 3. The gene *mtxA* comprises an ORF of 2784 nucleotides including stop codon.

The sequence of the cytotoxin gene was determined according to the procedure described in Example 18, using primers identified by the nucleotide sequences SEQ ID Nos: 23-26.

DNA sequence identified as SEQ ID NO: 30 represents the *M. bovis* RTXB gene *mtxB*. The nucleotide sequence of the *mtxB*

gene is shown in Figure 8. The gene *mtxB* comprises an ORF of 2215 nucleotides including stop codon.

DNA sequence identified as SEQ ID NO: 31 represents the *M. bovis* RTXC gene *mtxC*. The nucleotide sequence of the *mtxC* gene is shown in Figure 10. The gene *mtxC* comprises an ORF of 507 nucleotides including stop codon.

DNA sequence identified as SEQ ID NO: 36 represents the *M. bovis* RTX D gene *mtxD*. The nucleotide sequence of the *mtxD* gene is shown in Figure 12. The gene *mtxD* comprises an ORF of 1428 nucleotides including stop codon.

Corresponding protein products encoded by these genes are described above.

2. Cloning of *mbxA, mbxB, mbxC* and *mbxD* Genes and Expression of Recombinant Proteins

In order to produce highly purified recombinant cytotoxin peptides, portions of the cytotoxin gene were cloned into expression vectors according to procedures described in Example 18. An internal peptide and carboxy peptide were expressed corresponding to amino acids 438 through 713 and 643 through 927, respectively.

3. Confirmation of Recombinant Protein Identity

Identity of produced recombinant protein was confirmed by neutralization studies, described in Example 21 and in Figure 7.

Preparation of recombinant protein was done according to Example 18.

III. Biologically Derived or Recombinant Anti-*Moraxella bovis* Vaccines

A. Cytotoxin Vaccines

Vaccine is a biologically derived or recombinantly prepared agent useful for artificially acquired immunization in a host. The current invention describes the production of biologically derived and recombinant vaccines for active immunization of cattle and calves against IBK and includes the preparation of passive immune products for treatment of established infections.

The scope of the invention is, therefore, intended to include biologically derived or recombinantly prepared vaccines based on the antigens (a native or recombinant cytotoxin or a fragment thereof) of the invention.

The native vaccine is produced from isolated and partially purified native cytotoxin. The recombinant vaccine is produced by identifying the relevant cytotoxin or a fragment thereof, cloning them and expressing them using suitable vectors. This approach yields immunogens which are pure and reproducible in sufficiently large quantities to allow preparation of a vaccine for active immunization.

Recombinant vaccines are useful for immunization of the cattle or calves to produce the host's own antibodies against an *M. bovis* infection. Additionally, the recombinant vaccines may be used for production of passive immunotherapeutic agents.

These vaccines are also useful for primary protection against *M. bovis* infection in calves. Providing the herd with anti-*M. bovis* immunity decreases the risk for outbreaks of IBK in areas where the infection is prevalent.

In addition, *mbxA* DNA or RNA may be introduced into cattle so that propagation and/or expression of the encoded protein occurs in the host using a foreign expression system according to the methods known in the art.

The vaccine of the invention contains a cytotoxin identified by the invention, modified in such a way that it is incapable of producing the IBK symptoms but is capable of eliciting the production of specific protective antibodies against the disease when introduced in the body.

A DNA or RNA vaccine for prevention and treatment of infections caused by *M. bovis* is developed by utilizing newly identified and isolated DNA (SEQ ID NO: 1) and amino acid (SEQ ID NO: 2) sequences of the cytotoxin made by the *M. bovis* pathogen.

The antigen proteins used for preparation of vaccines correspond to cytotoxin or a cytotoxin fragment, which are identified by being a target of the polyclonal or monoclonal antibodies of the invention capable of preventing or ameliorating the disease.

A hybrid vector comprising a DNA segment that encodes the protein antigen able to bind selectively and specifically to anti-*M. bovis* antibodies operatively coupled to the vector can also be prepared and expressed. This includes preparation of recombinant vaccines using the viral expression vector outside of the host body and the preparation of DNA vaccines in procaryotic or eukaryotic hosts carrying the hybrid vector which may be introduced into the cattle or calf or a direct introduction of DNA or RNA into cattle or calf cells generating the host's own expression or translation of DNA or

RNA, respectively, and production of proteins eliciting appropriate antibodies.

B. DNA and RNA Vaccines

DNA or RNA vaccines for immunization against *M. bovis* are prepared as described in Science, 259:1745 (1993), hereby incorporated by reference. DNA or RNA vaccines for development of immunity for cattle and calves against IBK are produced according to the methods described ibid.

Briefly, animals are injected, preferably intramuscularly, with DNA vectors encoding the deactivated anti-*M. bovis* antigen DNA or RNA and the antigen is produced. Th produced antigen elicits its own immune responses in the form of a formation of a specific antibody against anti-*M. bovis* antigen, thereby providing its own (native) immunity and/or cell mediated responses.

C. Field Trials with Vaccines

Both *in vitro* and *in vivo* studies were performed in order to determine whether the native or recombinantly produced cytotoxin can be used as agent for provoking active immunity against *M. bovis* and for preparation of vaccines.

As described above, antibodies to both native partially purified and recombinantly produced cytotoxins were tested for their cytolytic and/or hemolytic inhibitory activity in neutralization studies described in Examples 7-13, and both were shown to be inhibited with antibodies raised against native or recombinant protein. Both proteins also induced production of antibodies that recognize cytotoxin/cytotoxin peptides.

Field trials were performed for testing both the partially purified native and recombinantly produced cytotoxin.

1. Native Cytotoxin Vaccines

M. bovis cytotoxin is a labile protein of which activity rapidly disappears when removed from the bacterial cell. This property previously prevented its complete characterization. To overcome this, a method for partial purification and stabilization of cytotoxin was developed and such partially purified and stabilized cytotoxin was used for preparation of a vaccine for field trials.

In order to determine suitability of such partially purified native *M. bovis* antigen (cytotoxin) for vaccination against *M. bovis*, two field trials were performed where the cytotoxin was administered as a vaccine formulated according to Example 23.

The first field trial was performed on 82 cattle to determine the suitability of the cytotoxin in a vaccine to prevent IBK. In this trial the vaccine was a partially purified cytotoxin formulated either in Quil A adjuvant or in an immunostimulating complex (ISCOM), a matrix that is composed of Quillaja saponins (Quil A), cholesterol, phospholipids and the antigen of interest. ISCOM based vaccines utilizing antigens of Bovine Herpes Virus-1, Bovine Virus Diarrhea Virus and Bovine Leukemia Virus are known to be successful immunogens in cattle.

Prior to the field trial testing the *M. bovis* cytotoxin vaccine, a preliminary study had shown that calves vaccinated with a cytotoxin vaccine developed higher tear IgA concentrations than calves vaccinated with a cytotoxin in oil

adjuvant. Results of the first field trial showed that calves receiving the cytotoxin vaccine had a significantly reduced requirement for treatment of IBK and had significantly reduced the severity of clinical IBK as compared to control calves.

To further evaluate the efficacy of an ISCOM vaccine using partially purified *M. bovis* cytotoxin, a second field trial was conducted according to Example 23.

Briefly, a group of 104 calves on a farm with a historically high (> 50%) incidence of IBK were randomly assigned to receive one of three different vaccine compositions: A) saline (controls); B) ISCOM-cytotoxin; and C) ISCOM alone (adjuvant).

Calves were vaccinated on days 0 and 21. The individual responsible for all subsequent examinations of the calves was blinded as to treatment group assignments. The calves were then examined once weekly throughout the summer.

In the saline control group A, total of 18 of 34 calves developed IBK, while only 7 of 35 calves in the ISCOM-cytotoxin group developed IBK. Fifteen of 35 calves that received adjuvant alone developed IBK. The cumulative number of calves with ulcers in one eye over time is shown in Figure 14. The cumulative number of calves that develop IBK was significantly less in the vaccinated group by the 10^{th} week and remained so until the end of the study. The number of calves with corneal ulcers during the study is presented in Figure 15.

The cumulative number of vaccinated calves with severe corneal ulcers (ulcer score > 1) was significantly lower in the antigen vaccinated calves than in the controls or in the adjuvant only group on observations. There were no

differences in cumulative ulcers in calves of the control or the adjuvant only groups.

The surface area measurements and the clinical scores of the corneal ulcers are shown in Table 6.

Table 6

Surface Area Measurements of the Corneal Ulcers and Clinical Scores of Vaccinated Calves and Controls

Group	Peak Surface area of First corneal ulcer	Surface area of corneal ulcer when first observed	Clinical score of corneal ulcer when first observed
Saline (n=34)	1.59±4.0(21.6)[v]	0.714±1.8(11.5)[v]	0.58±0.7[v]
Vaccine (n=35)	1.00±5.5(31.2[a,s]	0.082±0.2(9.2)[s,t]	0.71±0.8[a]
Adjuvant (n=35)	1.27±2.9(11.5)[v]	1.23±2.91(1.1)[v]	0.171±0.4

The numbers reflect the mean value ± the standard deviation. Numbers in parenthesis reflect the range of values.

Table 7

Descriptive Data Regarding Recurrences, Treatment and Peak Corneal Ulcer Scores

Group	No. Recurrences[a]	No. Calves treated	Peak CUS*
Saline (n=34)	5/18*	10*	
Vaccine (n=35)	3/7	4	
Adjuvant (n=35)	6/15	9	

* Differences were not significant (P>0.05)

[a] Numbers reflect number affected divided by the number of calves with corneal ulcers.

The number of calves in the saline, vaccine and adjuvant groups that received antibiotic treatment, and the number of recurrences was not significantly different (Table 7).

Intact iscoms were present in low concentrations in indirectly stained ultracentrifuged sediments that were examined by electron microscopy.

Beginning on the 8th observation week, and continuing thereafter until the 15th observation week, there were significantly fewer cumulative infections in calves in the vaccine group than in either of the control groups (Figure 14). There were no significant differences in numbers of cumulative infections between the calves that received adjuvant only and those of the controls.

Table 8

Cumulative Numbers of Calves with Corneal Ulcers at Each Observation

Observation Week	Group					
	Saline (n=34)		Vaccine (n=35)		Adjuvant (n=35)	
	All	≥2 CUS	All	≥2CUS	All	≥2CUS
0	0(0)	0(0)	0(0)	0	0(0)	0
1	0(0)	0(0)	0(0)	0	0(0)	0
2	0(0)	0(0)	1(2.9)	0	0(0)	0
3	1(2.9)	0(0)	1(2.9)	0	1(2.9)	1
4	1(2.9)	0(0)	1(2.9)	0	3(8.6)	1
5	2(5.9)	0(0)	4(11.4)	0	3(8.6)	1
6	3(8.8)	0(0)	4(11.4)	0	5(14.3)	1
7	5(14.7)	3(8.8)	4(11.4)	0	5(14.3)	1
8	7(20.6)	3(8.8)	4(11.4)	1	7(20.0)	3
9	9(26.5)	4(11.7)	4(11.4)	1	8(22.9)	3

10	12(35.3)	6(17.6)	4(11.4)	1	11(31.4)	5
11	13(38.2)	7(20.6)	4(11.4)	1	11(31.4)	6
12	13(38.2)	7(20.6)	4(11.4)	1	12(31.4)	7
13	14(41.1	8(23.5)	5(14.3)	1	14(34.3)	7
14	15(44.1)	8(23.5)	5(14.3)	3	15(40.0)	7
15	16(47.1)	10(29.4)	5(14.3)	3	15(42.9)	9
16	18(52.9)	10(29.4)	7(20.0)	4	15(42.9)	9

The native vaccine was found to be effective at preventing new cases of IBK and the vaccine afforded protection against natural exposure. The antigenic mass of this experimental vaccine was concentrated from 5 liters of supernatant from hemolytic, toxigenic *M. bovis*. The retentate of that process was diafiltered extensively, effectively removing any solubilized protein with a molecular mass <100,000 daltons. A companion study showed that the leukotoxin was concentrated and stabilized in the diafiltered retentate. This proved to be antigenic and immunogenic in the field study described herein.

As measured by decreased cumulative infection, and decreased numbers of affected calves, vaccination with the vaccine derived from cytotoxin induced a resistance to infection under field conditions.

Results from this and previous studies provided a rational basis for pursuing the development of the vaccine comprising the native cytotoxin. Unfortunately, the lability of the cytotoxin and the difficulty associated with purifying it in milligram quantities limited its usefulness for practical use. To overcome these obstacles, techniques of molecular cloning and biotechnology to study the *M. bovis* cytotoxin were undertaken as described above and in Examples

and the recombinant protein was used for preparation of recombinant vaccines.

2. Recombinant Vaccines

Recombinantly prepared cytotoxin was used for preparation of recombinant anti-*M. bovis* vaccine using procedure as described in Example 24.

For the preparation of vaccine, the *M. bovis* cytotoxin was expressed as described in Example 24, and expressed purified protein was mixed with the ISCOM following ISCOM matrix formation. The vaccine was prepared according to Example 24.

For vaccine production, ISCOM matrix preparation was mixed 1:1 (v/v) with the recombinant protein solution. Vaccine (2 ml) was used for primary vaccination and for follow-up booster inoculations. ISCOM marix adjuvant was used for control.

To determine if recombinantly derived *M. bovis* RTX toxin vaccine could prevent IBK, a vaccine trial was conducted during summer. In this trial, beef calves were randomly divided to receive saline, adjuvant alone (ISCOM matrix), or a recombinant *M.* bovis RTX toxin subunit vaccine. Vaccines were administered subcutaneously and calves were boostered 21 days later. The recombinant toxin vaccine contained the carboxy terminal 338 amino acids of MbxA. The calves were examined once per week for IBK through mid-September. Results indicated that fewer cases of IBK occurred in the recombinant vaccine group.

A total of 93 calves were enrolled in the study. Respective calf numbers in each vaccine group were: 29 CTRL (saline control); 31 ADJ (ISCOM MATRIX contact); and 33 VAC

(ISCOM matrix plus recombinant protein). At the end of the 20 week trial, a total of 47 calves developed corneal ulcers (50.5%). Respective numbers of ulcerated calves were 17 (54.8%), 17 (58.6%), and 13 (39.4%) for the CTRL, ADJ, and VAC groups. Figure 16 shows the cumulative proportion of calves with ulcers at each weekly interval. During weeks 4 and weeks 11 through 15, there were significantly fewer ulcerated calves in the VAC group relative to the CTRL group. At week 4, there were significantly fewer ulcerated calves in the VAC group relative to the ADJ group.

Results of this field trial clearly show that the vaccine comprising recombinantly derived cytotoxin provided immunity against *M. bovis* in vaccinated animals.

Prophylaxis of IBK with Antigen Vaccine

Using vaccines described above comprising either the purified native or recombinantly prepared cytotoxin antigen, the cattle and/or calves are vaccinated using a regimen typically utilized for immunization by vaccinating the animals at least twice in about a 3 week interval and with a booster vaccine at least once a year.

A method of prophylaxis of *M. bovis* infection comprises administering to the animal in need of such treatment a vaccine comprising the cytotoxin or recombinant cytotoxin of this invention capable of endogenous development of an inhibitory amount of anti-*M. bovis* antibodies.

V. Diagnostic Utilities

Cytotoxin of the invention and/or antibodies against *M. bovis* may also be used for diagnostic purposes, such as for detection of *M. bovis* carrier cattle.

In the simplest form, serum obtained from the cattle is reacted with monoclonal or polyclonal antibody raised against native or recombinant *M. bovis* cytotoxin. The antibody binding or forming complex with the antigen indicates the presence of antigen. Following techniques may be advantageously used for *M. bovis* antigen identification.

Antigen capture ELISA is a technique for identifying an antigen in a sample. In the case of M. bovis detection, it can be used to identify cytotoxin in tear samples from cattle or respiratory or vaginal secretions to identify carrier cattle. The technique utilizes an antibody directed against the antigen (cytotoxin)) which is fixed on a solid support. It then uses traditional ELISA techniques to develop a color change indicative of cytotoxin presence.

Diagnostic PCR utilizes primers, in this case, primers suitable for cytotoxin gene sequence, to amplify DNA from tissue samples, such as ocular, respiratory, or vaginal secretions, for example.

Fluorescence Resonance Energy Transfer (FRET) technology is also suitable to diagnose M. bovis carriers. This technology utilizes PCR primers linked to chemicals that fluoresce under certain conditions. If these primers detect the *mbxA* gene in sample DNA or bacteria, they emit certain detectable fluorescent spectra.

Method of diagnosing cattle that are carriers of *M. bovis* by use of antibodies (monoclonal or polyclonal) using an antigen capture ELISA.

Method to diagnose infection with hemolytic *M. bovis* utilizes diagnostic PCR of using DNA sequence data.

UTILITY

The current invention provides native or recombinant vaccines for immunoprotection against *M. bovis* infections and development of IBK in cattle and calves and methods for detection of latent *Moraxella bovis* infections.

EXAMPLE 1

Isolation of *Moraxella bovis* Bacteria

This example describes isolation of a cytolytic strain of *Moraxella bovis* T+ and a noncytolytic subculture T-.

A hemolytic, pathogenic strain of *Moraxella bovis* (T+) was isolated from a beef cow with infectious bovine keratoconjunctivitis. Cytolytic EPP and GLN 63 strains were furnished by Dr. G. W. Pugh (Ames, Iowa), and additional isolates were obtained from field specimens that were submitted to the laboratory for confirmation of bacterial identification. These additional isolates were recovered from clinically affected cattle located in regions of the United States that included southern Georgia (Tifton 1, Tifton 2), northern Georgia (GA 3), northern California (LS-2, LS-4, LS-6), southern California (LS-3, LS-8), Washington (LS-5), and North Carolina (LS-7). Isolate were identified and propagated as described in Acta Path. Microbiol. Scand., 80B, 629-640 (1972) and in Identification of Non-Enteric Gram-Negative Bacteria, US Dept. Health Education and Welfare Technical Report, Center for Disease Control, Atlanta, GA (1979).

EXAMPLE 2

Purification and Concentration of the Cytotoxin

This example describes the procedure used for purification and concentration of the cytotoxin when produced in *M. bovis* shaker cultures.

The cytotoxin was produced in broth shaker cultures that were inoculated with lawn cultures of *M. bovis*. The inoculum was harvested from the surface of 10, 20 hour sheep blood agar plate lawns of *M. bovis* with sterile cotton tipped applicators. The bacteria on the swab were suspended into 10 ml of heart infusion broth (Difco Laboratories, Detroit, MI) spiked with 1.5 mM $CaCl_2$.

Flasks were inoculated with the suspension, and were incubated at 35.5±0.5°C on a rotary shaker set at 200 oscillations per minute. The cultures were removed from the incubator when the optical densities (420 nM) reached 1.85. For purification, the cultures (living whole cells) were centrifuged for one hour at 13,000 x g (4°C) and the supernatants (centrifuged supernatants) were filtered through a sterile 0.2 μm polyethersulfone membrane (Gelman Sciences, Ann Arbor, MI).

Permeates from these filtrations (bacterial filter permeate) were concentrated approximately 100 fold by ultrafiltration, and further purified by diafiltration (diafiltered retentate) against 45 volumes of chilled (4°C) buffer consisting of 50 mM Tris (pH 8.2), 500 mM sodium acetate, 1.5 mM $CaCl_2$ and 20% glycerol. Diafiltration was discontinued after the optical density (280 nm) of the diafiltered permeate reached ≤0.01. Specimens collected at each stage of preparation were examined for hemolytic and leukotoxic activities.

Filters that were used for concentration and diafiltration, included cartridges of spiral wrapped, regenerated cellulose (100 kDa molecular weight cut-off, CH2PRS® system S1Y100 membrane, Amicon, Beverly, Ma), and flat

polyethersulfone membranes (300, or 500 kDa molecular weight cut-off, stirred cell, XM 300 or YM 500 membranes, Amicon).

Results indicated that there were no differences in the retention of the cytotoxin by the filters, but because of ease of use, and capacity, most studies were performed using the regenerated cellulose filter. Each filtration step was conducted according to the manufacturer's recommendations, which included 30 PSI operating pressure, 300 to 500 ml/minute flow rate, and a 5 to 10 PSI pressure differential.

Due to the length of time required for diafiltration, initial measurements of cytolytic activity did not commence until 16 to 20 hours after the broth cultures were first centrifuged. For stability studies, cytolytic activities of the refrigerated retentates and permeates were centrifuged. For stability studies, cytolytic activities of the refrigerated retentates and permeates were re-examined 7, 14, and 57 days later. Cytolytic activities of frozen (-70°C) samples were remeasured after 120 days. All measurements were made from at least duplicate experiments.

EXAMPLE 3

Cytotoxin Preparation for Neutralization Studies

This example describes the procedure used for preparation of cytotoxin for neutralization studies.

Moraxella bovis lawns were grown on trypticase soy agar plates supplemented with 5% sheep blood. After 24 hours of incubation, the bacterial cells were harvested by flooding the plate with 10 ml of Tris buffered saline solution (TBS $CaCl_2$ 50 mM Tris, 150 mM NaCl, 1.5 mM $CaCl_2$, pH 8.0), and suspending the bacterial growth with a sterile inoculating loop. The suspensions were collected from the surface of the agar, and then were centrifuged at 27,000 x G for one hour. After

centrifugation, the supernatants were harvested, filtered through 0.22 μM polyethersulfone membrane (Gelman Biosciences, Ann Arbor, Michigan), and tested for cytolytic activity.

EXAMPLE 4

Cytotoxicity Assay Using ^{51}Cr-Labeled Cells

This example describes the assay for determination of cytotoxicity, using cells labeled with ^{51}chromium.

A stable, tissue culture adapted cell line of bovine lymphocytes BL-3 cells, supplied by Dr. Gordon Thielen, University of California, Davis, were used as targets in the cytotoxicity assays. The cells were grown from frozen seed cultures at 37°C in 5% CO_2 in Liebovitz L-15 and Minimal Essential Media (1:1; v/v) with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 15% fetal bovine serum until sufficient density was achieved (3 to 4 days). The cells were harvested by centrifugation, and the pellets were washed 3 times in Dulbecco's phosphate buffered saline solution (DPBS). After the wash, the cells were resuspended in DPBS to a final concentration of 2 X 10^7/ml, labeled with 200 μCi of ^{51}Cr (1 hr, 37°C), and then washed three times in McCoys 5A buffer (Gibco/BRL, Grand Island, NY). After the final wash, the cells were diluted to a final concentration of 1 X 10^6/ml in McCoys 5A buffer.

For measurement of cytotoxicity, 500 μl of labeled BL-3 cells were added to 500 μl of sample and incubated for one hour at 37°C. After incubation, the cells were centrifuged and the radioactivity was measured in 500 μl of supernatant. Labeled cells (500 μl) in 500 μl of labeled cells lysed with Triton X-100 (Sigma, St. Louis, Mo.) served as respective negative and positive controls. The % lysis was calculated using the formula:

$$\% \text{ lysis} = \left[\frac{\text{counts per minute (CPM) of specimen- media CPM}}{\text{Triton X CPT- media CPM}}\right] \times 100$$

For calculations relating to specific activity, a unit of cytotoxic activity was defined as the amount of leukotoxin required to release 1% of the total amount of ^{51}Cr from the labeled BL-3 cells.

EXAMPLE 5

Cytotolytic Assay Using Non-Labeled Target Cells

This example describes the assay for determination of cytotoxicity using non-labeled target cells.

Cytolytic activity for bovine lymphocytes was also measured by assaying the release of lactate dehydrogenase (LDH). These tests were performed by washing BL-3 cells three times a diluting them to a final concentration of 5 x 10^6 cells/ml in DPBS, adding 100 μl of BL-3 cells to 100 μl of sample in 96-well polypropylene plates (Becton Dickinson, Franklin Lakes, NJ) and incubating the mix for 60 minutes (37°C) while being shaken for 30 seconds every 15 minutes. Duplicate measurements were made on each specimen.

After the incubation was completed, a 50 μl aliquot of supernatant from each well was transferred to a corresponding well in a new polystyrene plate (Costar, Cambridge, Ma), and 75 μl of 0.75 mM β-NADH in 100 mM potassium phosphate pH 7.2 was added. The change in absorbance (340 μm) was measured every 15 seconds using an automated reader.

After 50 measurements, lactate dehydrogenase activity was recorded in units/ml with units representing the reciprocal slope of a line plate of $\Delta A/\Delta T$. For validation, lytic activities of 0.22 μM filter sterilized culture supernatants were measured concurrently using ^{51}Cr release and LDH assays.

EXAMPLE 6

Neutralization Assay Using ^{51}Cr Labeled Cells

This example describes the assay used for neutralization with ^{51}chromium-labeled cells.

Prior to neutralization, sera were diluted 1:5 or 1:10 in TBS $CaCl_2$ or McCoys 5A buffer and 200 μl was mixed with 500 μl of specimen, refrigerated (4°C) for one hour, and then mixed with 500 μl of labeled BL-3 cells. The cells and sera were incubated (37°C) and rotated at 15 revolutions per minute for one hour before centrifuging each specimen and measuring the radioactivity in 500 μl of supernatant. For controls, dilutions of irrelevant rabbit sera, or fetal bovine serum was substituted.

EXAMPLE 7

Neutralization Assay Using Non-Labeled Target Cells

This example describes the assay used for neutralization with non-labeled target cells.

Cytolytic and hemolytic bacterial filter permeates were prepared by flooding agar lawn cultures of *M. bovis* with 10 ml of TBS $CaCl_2$, centrifuging the suspension and filtering the supernatants through sterile polyethersulfone membranes (0.2 μm average pore diameter). Heat inactivated sera were diluted in TBS $CaCl_2$. Aliquots (50 μl) of the bacterial filter permeates from T+ and T- were mixed with 50 μl of each serum dilution, and the mixture was refrigerated (4°C) for 60 minutes before the addition of 100 μl of diluted BL-3 cells. The cell-serum mixture was further incubated (37°C) for 60 minutes with gentle agitation for 30 seconds every 15 minutes. After incubation, 50 μl of the mixture was transferred to a new tissue culture plate to measure the cell free LDH activity.

EXAMPLE 8

Hemolysin Assay

This example describes conditions used for the hemolysin assay.

Bovine erythrocytes, harvested from 10 ml of heparinized blood of a normal cow, were packed by centrifugation and washed three times with TBS $CaCl_2$ buffer. For the hemolysin assay, 10 μl of packed erythrocytes were added to 500 μl of sera of 10-fold dilutions of sample. The mixtures were incubated for 6 hours (37°C) before final evaluation of hemolytic activity. Controls included washed erythrocytes in TBS $CaCl_2$ incubated with, or without 10% Triton X as respective positive and negative controls. For measurement of hemolytic activity of column fractions, a mixture of 10 μl of packed, washed bovine erythrocytes and 500 μl of sample, was incubated for 6 hours (37°C). After incubation, the cells were centrifuged, and the absorbance (455 μM) of the supernatant was measured. Units of hemolytic activity were calculated using the formula:

$$\text{Hemolytic units} = \frac{\text{Sample O.D.} - \text{Neg. Control O.D.}}{\text{100\% Lysis O.D.} - \text{Neg. Control O.D.}} \times 100\%$$

Percent hemolysis was calculated from hemolytic units of 100% lysis controls, and the specimen.

EXAMPLE 9

Immunoassay

This example describes conditions used for immunoassay.

The 96-well polystyrene plates (Nunc Maxisorp, Naperville, Ill.) were sensitized with 100 μl of solution containing 2 μg/ml diafiltered cytotoxin, refrigerated for 24 hour, and then washed with TBS $CaCl_2$ containing 0.05% Tween 20 (TBST). After washing, two-fold doubling dilutions of serum were added to the wells. The plate was then agitated gently

for 60 minutes at 37°C, and washed three times with TBST. After the final wash, either goat anti rabbit or rabbit anti bovine IgG labeled with 100 μl of alkaline phosphatase (Jackson Immunobiological, West Grove, Pa), diluted 1:25,000 in TBS $CaCl_2$, was added. Plates containing secondary antibodies were shaken continuously for 60 minutes (37°C), and then washed one more time with TBST. The color reaction was developed by the addition of 100 μl of 3.8 mM p-nitro phenyl phosphate (disodium salt) dissolved in a buffer consisting of 0.5 mM $MgCl_2$ and 50 mM Na_2CO_3 (pH 9.5). The plate was agitated gently for 60 minutes at 37°C and then absorbance (400 μm) was measured using an automated plate reader (Dynatech MR700, Chantilly, Va).

EXAMPLE 10

Chromatography of Diafiltered Retentates

This example describes conditions used for chromatography diafiltered retentates.

Diafiltered retentates of T+ and T- were chromatographed through a 10 x 300 mm Superose 6HR gel filtration column (FPLC, Pharmacia Biotech, Uppsala, Sweden) that was equilibrated with 50 mM Tris, 0.5 M sodium acetate, 1.5 mM $CaCl_2$ pH 8.0. The column was loaded with 200 μl of diafiltered retentate and was eluted with buffer at a maximum flow rate of 0.5 ml/min^{-1}. Absorbance (280 nm) of column effluent was measured. Fractions (3.5 ml) were collected, and examined for cytotoxic and hemolytic activity, and for endotoxin content.

EXAMPLE 11

Production of Antibodies

This example describes procedure used for the production of antibodies against *M. bovis* cytotoxin.

New Zealand White rabbits were given 0.5 ml of either diafiltered retentate (50 μg protein), gel filtration purified cytotoxin (10 μg protein) or permeates from the 0.22 μM filtration of T+ or T- broth cultures.

Immunizing preparations were mixed 1:1 (v/v) in Freunds complete adjuvant for the primary injection and with incomplete Freunds adjuvant for the booster, 21 days later. The rabbits were exsanguinated three weeks after the booster was given. Convalescent serum from a Holstein calf with clinically apparent infectious bovine keratoconjunctivitis and fetal bovine serum (Hyclone Laboratories, Logan, Utah) were used as respective positive and negative controls.

EXAMPLE 12

General Methods

This example describes or gives reference to general methods used in these studies.

Measurement of 2-Keto-3-Deoxyoctonate (KDO)

The concentration of KDO was measured in diafiltrates, and cytolytic Superose 6HR column fractions using a hydroperiodate oxidation assay as described in Handbook of Micromethods for the Biological Sciences, Van Nostrand Reinhold, New York (1976).

Measurement of Endotoxin

The concentration of endotoxin in cytolytic Superose 6HR column fractions was measured semiquantitatively using a commercially available timed gel formation limulin assay (Sigma, St. Louis, Mo.).

Electrophoresis

Proteins were examined using the discontinuous polyacrylamide gel electrophoresis method described in Nature, 227:680-685 (1970).

To reduce non-specific binding, blots were blocked in a buffer containing 0.1% SDS, 50 mM Tris and 150 mM NaCl, pH 7.5, containing either 1.5% goat serum, or 0.5% teleost gelatin (Sigma Inc., St. Louis, Mo). The blocked, washed membranes were incubated with antiserum for 12 hours, and then were washed four times in TBS. After the final wash, blots were incubated with either 1:2500 dilution of goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase, or with 0.5 μCi of I^{125} labeled protein A in TBS. The alkaline phosphatase labeled blots were incubated for 12 hours, after which they were washed four times (10 minutes per wash) with a buffer containing 100 mM Tris, 5 mM Tris, 5 mM $MgCl_2$ and 100 mM NaCl (pH 9.5). After the final wash, the blots were developed using nitro blue tetrazolium and bromo-chloro-indonyl-phosphate as described in Antibodies: A Laboratory Manual, 1st Ed., Cold Spring Harbor, Cold Spring Harbor Laboratories, New York (1988). The A-I^{125} protein A-I^{125} labeled blots were incubated for 2 hours, and then washed 3 times, 4 hours per wash, in TBS containing either 1.5% goat serum or 0.5% teleost gelatin. After washing, the blots were air dried and autoradiographed.

Protein Quantitation

Protein concentrations were measured using the Lowry protein precipitation method (Protein Assay Kit, Sigma Inc., St. Louis, Mo.).

EXAMPLE 13

Corneal Epithelial Cell Cultures

This example describes procedures used for culturing corneal epithelial cells.

Bovine eyes were collected at slaughter, and the corneal epithelium was harvested by keratectomy. An ophthalmic

solution containing neomycin, polymyxin B and gramicidin (Schein Pharmaceuticals, Port Washing, NY) was applied to the corneas prior to keratectomy in order to reduce the bacterial contamination.

The corneal epithelium was dissected from the stroma, and immersed in 50 ml of Minimal Essential Medium (MEM) containing D-valine, penicillin G (100 IV/ml), streptomycin (100 μg/ml), gentamicin sulfate (100 μg/ml), and amphotericin B, (250 μg/ml). Neutral protease, (Dispase II, Boehringer Mannheim, Indianapolis, Inc.) was added in order to initiate separation of the epithelial cells from the remaining stroma.

After 2 hours of incubation, the epithelial sheets were washed three times in fresh media, and were placed epithelial side down, in wells of sterile polystyrene plate. The cultures were then immersed in 2 ml of media containing MEM-D-valine and 10% fetal bovine serum, and incubated in an atmosphere of 5% CO_2 (37°C) until plaques of adherent corneal cells were observed on the bottom of the plate. The epithelial sheets were removed from the plates at that time. These cell cultures were incubated for at least 48 additional hours until they formed an adherent monolayer.

In order to verify the epithelial origin of the adherent cells, sample monolayers were stained with an anti-keratin, epithelium specific monoclonal antibody furnished by Dr. Rheen Wu, University of California, Davis. After 1 hour, the cultures were washed with PBS containing 0.05% Tween 20, and a fluorescein isothiocyanate labeled goat anti-mouse antibody (Organon Teknika, Durham, NC) was applied for 1 hour. The unbound secondary antibody was then washed from the cells using PBS with 0.05% Tween and the monolayer was examined using a fluorescence microscope.

For measurement of cytolytic activity using corneal cell targets, plates containing confluent epithelial cells were washed three times with DPBS to remove the media and then were incubated with 1.0 ml of diafiltered cytotoxin. Respective negative and positive controls included cells with 1.0 ml of DPBS or with 1.0 ml of DPBS to which 10% Triton X was added. Assays were performed in duplicate. After one hour of incubation (37°C), the culture fluid was removed and the amount of LDH released from the cells was measured as described in DNAS, 48:2123-2130 (1962).

EXAMPLE 14

Bacterial Strains and Culture Conditions for Cloning

This example illustrates isolation of pathogenic strains of *M. bovis* and culturing conditions for cloning.

Hemolytic, pathogenic strains of *Moraxella bovis* (strains T+ and Tifton I) were isolated from 2 beef cows infected with IBK. A nonhemolytic mutant (T-) arose spontaneously from T+ subcultures. Isolates were identified as *M. bovis* by colony morphology and biochemical criteria. *Moraxella bovis* was propagated on 5% sheep or cow blood agar plates and strains were stored frozen in 50% skim milk-50% glycerol media at -80°C. Cloning competent *E. coli* strains TOP10 (Invitrogen Corp., Carlsbad, CA), DH5α (Life Technologies, Inc., Rockville, MD), and BL21 (DE3) (Novagen, Madison, WI) were propagated in LB broth or on LB agar (1.5% agar; Difco Laboratories, Detroit, MI). Antibiotic selection of *E. coli* was made using ampicillin (100 μg/ml).

EXAMPLE 15

Western Blot Analysis

This example describes conditions used for Western blot analysis.

Diafiltered retentate was buffer exchanged into FPLC buffer (50 mM Tris; 1.5 mM $CaCl_2$; 0.9% NaCl; 5% glycerol; pH 8.0) using PD-10 columns (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), concentrated 10-15 fold (Centriprep-10; Millipore, Beverly, MA), filtered through a 0.2 μm filter (Sterile Acrodisc 13; Gelman Sciences), and chromatographed on a Superose 6 gel filtration column (Amersham Pharmacia) in FPLC buffer at room temperature. Column void volume fractions from multiple chromatography runs were pooled and concentrated 5-10 fold (Centriprep 10; Millipore).

For analysis, samples were mixed with an equal volume of 2X loading buffer containing 62.5 mM Tris, pH 6.8, 0.7 M β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA), 20% glycerol, 4.1% sodium dodecyl sulfate (SDS), and 0.2 mg/ml bromophenol blue, heated to 95°-100°C for 5 minutes, electrophoresed using SDS-PAGE (3.9% stacking/7.5% running), transferred to Immobilon-P (0.45 μM pore size; Millipore), and incubated overnight at room temperature on a rotating shaker in blocking buffer (TSN buffer; 20 mM Tris, 0.9% NaCl, 0.1% Tween 20 (pH 7.4) with 5.5% teleostean gelatin (Sigma, St. Louis, MO)). Following incubation, fresh blocking buffer containing rabbit anti-column fraction serum (1:400 dilution) was added and blots were incubated overnight at room temperature on a rotating shaker. The blots were washed 3 times in TSN buffer, then immersed in a solution containing 0.5 μCi of ^{125}I-labeled Protein A (NEN Life Science Products, Inc., Boston, MA) per 20 ml TSN buffer, incubated overnight at room temperature on a rotating shaker, washed and autoradiographed (Hyperfilm MP; Amersham).

EXAMPLE 16

Amino Acid Sequencing

This example describes conditions used for amino acid sequencing.

The concentrated void volume fractions from gel filtration chromatographed diafiltered retentates from T+ were electrophoresed on a SDS-PAGE gel and stained with Coomassie blue. A 70 kDa band that had previously been identified on Western blots as being unique to T+ was excised, digested with trypsin, and tryptic peptides were fractionated by HPLC. Two peptides designated #23 and #26 were selected for N-terminal amino acid sequencing using Edman degradation chemistry. Amino acid sequencing was performed at the Protein Chemistry Laboratory, University of California, Davis (ABI 470A amino acid sequencer; PE Biolsystems, Foster City, CA).

EXAMPLE 17

Determination of DNA Sequence of Cytotoxin Gene

This example describes conditions used for determination of DNA sequences of *mbxA* gene.

All DNA manipulations were performed as recommended by the manufacturers of the enzymes or by using standard published methods. Genomic DNA from *M. bovis* Tifton 1 was isolated as described in Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989).

Plasmid DNA was isolated with Qiagen plasmid minipreps (Qiagen, Valencia, CA). The DNA sequence of the complete cytotoxin gene was obtained by sequencing PCR products directly or PCR products cloned into pCR2.1-TOPO (Invitrogen) with a TOPO TA cloning kit (Invitrogen). Degenerate PCR primers designated primer 23A, depicted by a sequence 5'-AAY

AAA GAR TTR GAR GCN GAR-3' (SEQ ID NO: 23) and 26A, depicted by a sequence 5'CCY TCN CCR CTR TGR AAD ATR TCR TTR AAT TT-3' (SEQ ID NO: 24) were designed by reverse translating the amino acid sequences of peptides #23 and #26, respectively, primers C-down, 5'ATH GAY TGG ATH GCN CCN TTY GGN GAY-3' (SEQ ID NO: 25) and B-up, 5'-ACT TTA TCC ATC ACR ACT TGR AAR AA-3' (SEQ ID NO: 26) were designed by reverse translating conserved regions of RTXC and B genes of other bacteria.

PCR amplifications were performed with Taq polymerase (Life Technologies) using 30 cycles of 30 seconds each at 95°C, 1 minute at 55°C, and 45 seconds (primers 23A/26A) or 2 minutes (primers C-down/B-up) at 72°C. PCR products were analyzed by agarose gel electrophoresis. Automated DNA sequencing was performed by the DNA sequencing facility, University of California, Davis. Additional primers were designed as needed for sequencing of overlapping fragments on both strands; the DNA sequences of overlapping fragments were assembled with MacDNASIS (Hitachi Software Engineering Co., Ltd., Yokohama, Japan) to yield the final complete sequence.

EXAMPLE 18

Expression of Recombinant Peptides

This example describes expression of recombinant peptides.

Internal peptide

The PCR product amplified by primers 23A/26A was cloned into pCR2.1-TOPO (Invitrogen) and the resulting recombinant plasmid was digested with EcoRI. The appropriate fragment was gel purified (QIAquick gel extraction kit, Qiagen) and ligated into pProEXHTa (Life Technologies) digested with EcoRI to yield an expression construct that would direct synthesis of the cytotoxin gene from amino acids 438 through 713. This

expression plasmid was introduced into *E. coli* DH5α, cells were grown to an OD_{600} of 0.6, and expression was induced by adding isopropylthio-β-galactoside (IPTG; Life Technologies) to 1 mM. Induction was continued for 4-6 hours and cells were harvested by centrifugation.

The expressed proteins formed inclusion bodies that were purified as described in Methods Enzymol., 153:461 (1987). Purified inclusion bodies were then solubilized in buffer containing 20 mM Tris (pH 8.0), 1 mM EDTA, and 6 M urea and chromatographed on a SuperDex 200 gel filtration column (Amersham Pharmacia). Peak fractions were pooled, dialyzed against phosphate buffered saline (PBS) and stored at -20°C. Following removal of urea, the purified protein formed a precipitate. This protein was designated internal peptide.

Carboxy peptide

The complete cytotoxin gene was amplified from genomic DNA of *M. bovis* Tifton I with primers designated NdeI-start (5'-GAT CAT ATG TCC AAT ATA AAT GTA ATT AAA TCT AA-3') SEQ ID NO: 27 and B-up #2 (5'ATC ACT AGT TCC ATA ATC TAT AAC CAA TGA-3') SEQ ID NO: 28. This primer pair amplified *MbxA* from 6 bases before the ATG start codon to 64 bases past the termination codon and introduced an NdeI restriction site overlapping the ATG start codon. The PCR conditions were as described above except that the elongation step was for 1 minute at 72°C.

The PCR product was cloned into pCR2.1-TOPO (Invitrogen). The resulting recombinant plasmid was digested with XhoI-EcoRI and blunt ended with Klenow polymerase (Life Technologies). The appropriate fragment was gel purified, then cloned into blunted ended, BamHI digested and Klenow blunt ended pT7-7 to

yield an expression construct that would direct synthesis of the cytotoxin gene from amino acids 643 through 927.

Recombinant plasmids were transformed into *E. coli* DH5α, and subsequently purified (Qiagen miniprep kit; Qiagen) prior to transformation into *E. coli* strain BL21 (DE3) (Novagen, Madison, WI) for expression. The cells were grown and induced as described above to produce the recombinant protein, except that for protein purification, inclusion bodies were solubilized in buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA, and 8 M urea, and chromatographed on a Mono Q column (Amersham Pharmacia). Peak fractions were identified by SDS-PAGE, pooled and then chromatographed on a SuperDex 200 gel filtration column. Peak fractions from the SuperDex 200 column were pooled, dialyzed against PBS, and stored at -20°C. This protein, which remained soluble following dialysis, was designated carboxy peptide.

EXAMPLE 19

Preparation of Rabbit Antisera

This example describes preparation of rabbit antisera for recombinant cytotoxin identification and confirmation.

Antisera was prepared by immunizing New Zealand White rabbits with either the pooled void volume fractions or Superose 6 gel-filtration chromatographed diafiltered retentates of *M. bovis* T+ mixed 1:1 (v/v) with Freund's complete adjuvant. Booster vaccinations were performed 21 days later with either the pooled column fractions or culture filtrates mixed 1:1 with Freund's incomplete adjuvant, and serum was harvested 21 days post booster. Sera against the pooled column fractions was designated rabbit anti-column fraction. The sera against the culture filtrates was designated rabbit anti T+. This antisera neutralized

hemolytic and cytolytic activity of partially purified *M. bovis* cytotoxin.

Polyclonal rabbit antisera to the internal (amino acids 438 through 713) and carboxy (amino acids 643 through 927) peptides expressed from *MbxA* were prepared commercially (Antibodies Incorporated, Davis, CA). Before use in immunoblots, this antisera was preabsorbed with an *E. coli* DH5α lysate. Sera were heat inactivated at 56°C for 1 hour prior to lysis neutralization assays.

EXAMPLE 20

Demonstration of Cytotoxin in Culture Supernatants

This example describes conditions used for demonstration of the presence of cytotoxin in culture supernatant.

Moraxella bovis T+, T-, or Tifton I was inoculated into LB broth containing 1.5 mM $CaCl_2$ and incubated for 3-6 hours with shaking at 250 rpm at 35°C. Following incubation, cultures were centrifuged and the supernatants were harvested and analyzed by SDS-PAGE/transblotting, using rabbit antiserum against the internal or carboxy peptide as the primary antibody.

EXAMPLE 21

Lysis Neutralization Assays

This example describes assays performed to study cytolysis neutralization and hemolysis neutralization.

Serum test samples were diluted 1:64 in TBS $CaCl_2$ buffer (50 mM Tris, 150 mM NaCl, 1.5 mM $CaCl_2$, pH 7.4) and an equal volume of either TBS $CaCl_2$ buffer or carboxy peptide diluted in TBS $CaCl_2$ buffer (0.1 mg/ml) was added. The samples were incubated at room temperature for 30 minutes, then at 37°C for 30 minutes, and then centrifuged at maximum speed in a microcentrifuge for 10 minutes. The supernatants were then

harvested and mixed with an equal volume of Tifton I diafiltered retentate diluted 1:32 in TBS $CaCl_2$ buffer. The test samples were incubated for 1 hour at 4°C after which hemolysis or cytolysis neutralization assays were performed as described below.

For hemolysis neutralization assays, 500 μl of 1% (v/v) suspension of washed and pelleted bovine erythrocytes in TBS $CaCl_2$ buffer was added to 500 μl of each test sample, incubated for 6 hours at 37°C, the samples were inverted once, centrifuged for 1 minute at maximum speed in a microcentrifuge, and 350 μl of supernatant was transferred to a 96 well microtiter plate for determination of the OD_{455} (Spectra Max 250, Molecular Devices Corporation, Sunnyvale, CA). Respective negative and positive controls were TBS $CaCl_2$ buffer or Tifton I diafiltered retentate diluted 1:32 in TBS $CaCl_2$ buffer. A control for the carboxy peptide in TBS $CaCl_2$ buffer was also included. The percent hemolysis was determined using the formula:

$$\% \text{ hemolysis} = \left[\frac{\text{sample OD-lysis negative control OD}}{\text{positive control OD-lysis negative control OD}}\right] \times 100$$

The percent neutralization was calculated by subtracting the percent hemolysis from 100. The results reflect the means from three experiments.

The target cells for cytolysis neutralization assays were labeled bovine lymphocytes (BL-3 cells); supplied by Dr. Gordon Thielen, University of California, Davis. These were prepared by growing cells from frozen stock cultures at 37°C in 6% CO_2 in Liebovitz L-15 medium (Life Technologies) and Minimal Essential Media (Life Technologies) (1:1 v/v) supplemented with penicillin (50 U/ml), streptomycin (50 ug/ml) (Life Technologies), and 15% heat inactivated fetal bovine serum (Hyclone, Logan UT). Cells were harvested by

centrifugation, washed 3 times in PBS, resuspended in McCoys 5A media (Life Technologies) to a final concentration of 2 X 10^7/ml, labeled for 1 hour at 37°C with 200 μCi of ^{51}Cr (NEN Life Science Products) while rotating at 20 revolutions per minute, and then washed 3 times in McCoys 5A media. Following the final wash, cells were diluted in McCoys 5A media to a final concentration of 4 x 10^5/ml.

For cytolysis neutralization assays, 500 μl of labeled BL-3 cells were added to 500 μl of each test sample, incubated for 1 hour at 37°C while rotating at 20 revolutions per minute, pelleted in a microcentrifuge, and the radioactivity in 500 μl of supernatant was determined. Negative and positive controls were as described above for hemolysis neutralization assays. The percent cytolysis was determined using the formula:

$$\% \text{ cytolysis} = \left[\frac{\text{specimen counts per minute (cpm) - negative control cpm}}{\text{positive control cpm - negative control cpm}}\right] \times 100$$

The percent neutralization was calculated by subtracting the percent cytolysis from 100. The results reflect means obtained from three experiments.

EXAMPLE 22

Amino Acid Sequence Analysis

This example describes methods used in amino acid sequence analysis.

The relationships between the deduced amino acid sequences and published protein sequences were determined with BLAST database searches. Multiple sequence analyses, alignments, and homology calculations were obtained using the blosum 62 amino acid substitution matrix (SEQWEB software (version 1.1); Genetics Computer Group, Madison, WI). The nucleotide sequence for the *M. bovis* cytotoxin gene has been submitted to the GENBANK database under accession number AF205359.

EXAMPLE 23

Field Trials With Native Cytotoxin Vaccine

This example describes conditions used in field trials with native cytotoxin based vaccine.

Experimental Subjects

Cattle for the study were part of the herd at the University's Sierra Foothills Field station. The ranch was located in the foothills of the northern Sierra Nevadas at an altitude ranging between 800 and 2400 feet. The subjects included 104 Hereford Angus crossbred yearling calves. The calves, which were born during November and December of the previous year, weighed between 90 and 300 kg, and were composed of equal numbers of females and castrated males. Calves were commingled into a single group and were pastured on either of 2 irrigated fields. Other than the pasture forage and trace mineralized salt licks, cattle were not supplemented during the summer. The calves were housed out of doors on irrigated pastures on approximately 100 acres. Pasture forages were composed of Bermuda, fescue, oats, and rye grasses, and smaller amounts of trefoil and clovers, and other plants that were indigenous to the area. For the previous decade, yearling calves at the ranch had an annual prevalence of IBK that exceeded 50%.

Study Design

The study was conducted between the dates of May and September. In the May examination, calves were prospectively and randomly allocated to one of 3 groups designated A, B or C. For examination, the calves were restrained in a squeeze chute, and fluorescein dye was instilled into each eye prior to illumination with a light source. Eyes were assigned a clinical score ranging between 0 and 3 with 3 representing an eye that had ruptured, and had expelled the contents. Criteria for lower clinical ocular scores included: 0 = no conjunctivitis or

corneal ulcer; 1 = conjunctivitis with a corneal ulcer having a maximum diameter that was $\leq$ 0.5 cm, and 2 = a corneal ulcer having a maximum diameter that was > 0.5 cm. Eyes with corneal ulcers were photographed during each examination. Each photographic image included a ruler held on the lower eyelid.

After application of the fluorescein dye, eyes with corneal ulcer scores $\geq$ 1 were photographed at a constant magnification, and the image was projected onto paper and traced. The surface area of each traced image was measured planimetrically as described. During the first examination in April (Day 1), calves with normal eyes were randomly assigned to one of 3 groups. Calves of the first group (n=34) were given 2 ml of sterile saline solution. Calves of the second group (n=35) were given 2 ml of a cytotoxin enriched, sterile Quil A adjuvanted vaccine. Calves of the third group (n=35) were given 2 ml of a mixture of sterile adjuvant and saline (1:1, v/v). All injections were made subcutaneously in the neck. Calves were boostered with the corresponding product in June. Thereafter, clinical examinations were performed weekly for 15 weeks.

Personnel responsible for administering the vaccine, evaluating the clinical scores and for measuring the corneal ulcer diameters had no knowledge of the group assignment of the calves. Each week, calves with corneal ulcer scores ranging between 0 and 1 in either eye were returned to the common pasture. Calves with ulcer scores of 2 or 3 were treated with florfenicol (Nuflor®, 20 mg/kg of body weight intramuscularly) and were housed in a corral until they were retreated 48 hours later. After the second florfenicol treatment, the calves were moved back to their original pasture and were observed weekly for the remainder of the study.

Vaccines

Calves of group A were given 2.0 ml of a vaccine composed of Quil A adjuvanted, diafiltered concentrated cell free

cytotoxin from a toxigenic, hemolytic, non piliated strain of *Moraxella bovis*. Calves of the second group were sham vaccinated with Quil A adjuvant in phosphate buffered saline solution. The third group of calves received 2.0 ml of sterile saline solution. The original piliated strain of *M. bovis* was derived from the eye of an infected cow located in northern California and was identified using biochemical tests described previously. Non piliated isolates were identified during repeated subcultures on blood agar plates. These isolates, devoid of pili, were smooth, did not pit the surface of the agar, and remained homogeneously suspended in broth cultures. Lack of piliation was confirmed using electron microscopy.

Stocks of the pilin negative isolate were stored frozen (-80°C). For vaccine production, stocks were thawed at room temperature and inoculated onto a bovine blood agar plate lawns. After 18 hours of incubation (37°C), the lawns were harvested and were suspended in flasks containing brain heart broth (1 lawn per 500 ml) supplemented 1.5 mM of $CaCl_2$. The flasks were shaken (200 rpm) for 5 hours (37°C). After incubation, the broth cultures were centrifuged for one hour at 13,000 x g (4°C) and supernatants were harvested and were filter sterilized (0.2 μM average pore diameter; Gelman Sciences, Ann Arbor, Mi). The sterile supernatant was concentrated approximately 40-fold to a final volume of 200 ml by ultrafiltration over a spiral wrapped membrane (molecular weight cut off of 100,000 daltons) of regenerated cellulose (S1Y100 CH2PRS membrane, Amixon Inc, Beverly, MA). The concentrated retentate was then diafiltered using 50 volumes 50 mM Tris, pH 8.2, 50 mM sodium acetate, 1.5 mM $CaCl_2$, and 20% glycerol (v/v).

Diafiltration was discontinued when the optical density of the permeate was < 0.01 optical density units. Approximately 200 ml of diafiltered retentate was collected, and the protein content was measured using the Lowry method. The specific

cytotoxic and hemolytic activities of the diafiltered retentates were greater than those of the concentrated permeates.

The experimental vaccine preparation was made by diluting the diafiltered retentate to achieve a final concentration of 0.8 mg/ml, and then adding 0.25% (w/v) MEGA-10 (Cal Biochem, San Diego, CA). For the adjuvant control vaccine, an equivalent volume of Tris acetate calcium chloride, glycerol buffer was used in place of the diafiltered retentate. To both solutions, Quil A (Iscotec, Sweden) was added to a final concentration of 0.1 %. The mixtures were then placed in dialysis membranes with 2000 kDa molecular weight cut off, and were dialyzed against repeated changes of sterile Dulbecco's phosphate buffered saline solution (GIBCO BRL, Grand Island, New York) for 48 hours at 4°C. After 48 hours, the contents of the dialysis tubing were filter sterilized, and kept refrigerated (4°C) until administered to the subjects 48 hours later.

Statistical evaluation of data

Data including number of calves in each group that were infected, on each date, cumulative numbers of affected calves, and number of corneal ulcers in calves of each group during the weekly evaluation, were compared using the Chi squared analysis. In order to avoid statistical errors caused by repeated measurements in eyes that had more chronic ulcers, data from each week were evaluated independently. Corneal ulcer surface measurements of the calves were compared among groups using the analysis of variance test. Clinical score data were compared across the 3 groups each week using the Kruskal-Wallis test, with inter-group comparisons being made with the Mann-Whitney rank sum test. The data were considered to be significantly different when the p value was ≤ 0.05. Eyes with recurrences of corneal ulcers were not included in the statistical analyses, but the numbers of recurrences were noted and compared between the 3 groups using the Chi squared analysis.

EXAMPLE 24

Field Trials With Recombinant Vaccine

This example describes conditions used in field trials with recombinant anti-*M. bovis* vaccine.

Experimental Subjects

The study population consisted of calves at the University's Sierra Foothills Field Station (SFS), located in Brown's Valley, CA. The terrain consisted of Sierra foothill range pasture. The animals were predominantly Angus or Angus-Hereford crossbred calves. Calves weighed between 98 and 292.7 kg. The study population consisted of 20 intact males, 71 steers, and 2 heifers.

Study Design

Pre-enrollment Examination

Each calf was restrained in a head catch and both eyes were examined for the presence of corneal opacities indicative of prior ocular disease. Only calves with normal corneas were selected for the study. On day 0, calves were administered a 2 ml subcutaneous injection of either saline (CTRL group), ISCOM matrix adjuvant alone (ADJ group), or recombinant M. bovis cytotoxin-ISCOM matrix adjuvant (VAC group). Calves were randomly assigned to one of the three groups by use of a blocked randomization scheme. Individuals administering vaccine were unaware of the vaccine contents administered. Booster vaccinations were repeated on day 21.

Post-enrollment Examination

Each calf was examined weekly for 20 weeks following the primary vaccination. During these examinations, eyes were examined for the presence of corneal opacities. All eyes with corneal opacification were stained with fluorescein dye, irrigated with sterile isotonic saline solution, and assigned a corneal ulcer score (CUS) of 0, 1, 2, or 3 1 based on the widest diameter of the ulcer as determined by holding a ruler

next to the eye. The scoring criteria were: 0 = no ulcer; 1 = a corneal ulcer with the widest diameter ≤ 5 mm: 2 = a corneal ulcer with the widest diameter > 5 mm: and 3 = a perforating corneal ulcer. Ulcerated eyes were photographed with a ruler held next to the eye for determination of the corneal ulcer surface area measurement (SAM) as described below. Any calves with a CUS of 2 or greater were administered florfenicol (40 mg/kg subcutaneously). To minimize iatrogenic infections, garments including plastic aprons, obstetrical sleeves, and rubber gloves were worn and were rinsed in 1% chlorhexidine solution after each animal was examined.

Bacterial Strains and Culture Conditions

Cloning competent *E. coli* strains TOP10, DH5α, and BL21 (DE3) were propagated in LB broth or on LB agar (1.5% agar). Antibiotic selection of *E. coli* was made using ampicillin (100 ug/ml).

Expression of recombinant *M. bovis* cytotoxin

The carboxy terminus of the M. bovis cytotoxin gene was amplified with primers SNP down (5'-AAT GAC GAT ATC TTT GTT GGT CAA GGT AAA-3') and LNP2 (5'-TAG TAA ATT AAA TNA CTW AAC ACT-3'). PCR amplifications were performed with Taq polymerase using 30 cycles of 30 seconds each at 95°C, 1 minute at 55°C, and 45 seconds at 72°C, followed by a 10 mm incubation at 72°C. The PCR product was cloned into pCR2.1-TOPO with a TOPO TA cloning kit and the resulting recombinant plasmid was digested with EcoRI. The appropriate fragment was gel purified (QIAquick gel extraction kit) and cloned into blunted ended, SmaI digested pT7-7 to yield an expression construct that would direct synthesis of the cytotoxin gene from amino acids 590 through 927. Recombinant plasmids were transformed into *E. coli* DH5α, and subsequently purified prior to transformation into *E. coli* strain BL21 (DE3) for expression. Cells were grown to an OD_{600} of 0.8, and expression was induced by adding isopropylthio-β-

galactoside to 1 mM. Induction was continued for approximately 9 hours and cells were harvested by centrifugation. The expressed proteins formed inclusion bodies that were purified. Purified inclusion bodies were then solubilized in a buffer containing 4 M urea, 0.25% Triton X-100, 5 mM Tris-HCl (HCl pH 7.5) and 1 mM EDTA and chromatographed (Mono Q column; Amersham Pharmacia). Peak fractions were identified by SDS-PAGE, pooled and protein was quantitated with a BCA kit using a bovine serum albumin standard. The protein solution was sterile filtered and dialyzed against PBS to remove urea and stored at -20°C. Following dialysis the purified protein precipitated. PBS was added to the dialysate to make the final protein concentration 0.5 mg/ml.

Vaccine Preparation

ISCOM matrices were prepared as described in Current Protocols in Immunology, Caligan, John E., Ed. New York, Greene Publication Associates and Wiley-InterScience, except that no protein was incorporated into the ISCOMs. Instead, the purified recombinant protein was mixed separately with ISCOMs following ISCOM matrix formation. A lipid mixture containing 50 mg/ml each of phosphatidylcholine and cholesterol was made by dissolving 100 mg of each in 1 ml of chloroform and combining both solutions. This lipid mixture was then added to a 20% solution (w/v) of decanoyl-N-methylglucamide (Mega 10) made by dissolving 2 g Mega 10 in 10 ml distilled water. The resulting solution was added to phosphate buffered saline (PBS; pH 7.4) (0.1 cc lipid mixture per 1 ml PBS) and the chloroform was removed by heating the solution to 45°C under vacuum until the solution cleared. Quil A was then added to a final concentration of 1 mg/ml. The solution was transferred to dialysis tubing (2000 MW cut-off) and dialyzed extensively for 3 days in 50 mM Tris-HCI, 0.001% thimerosal, pH 7.5 at room temperature. Thimerosal was removed by dialysis against PBS (pH 7.4) for 24-36 hours at 4°C.

Following dialysis the solution was sterile filtered (0.45 or 0.2 μM pore size) and stored at -20°C until use. The success of ISCOM matrix formation was confirmed by electron microscopy performed at the Department of Medical Pathology, School of Medicine, UC Davis.

For vaccine production, 145 ml of ISCOM dialysate was made to 180 ml with PBS (pH 7.4). For the adjuvant control vaccine (ADJ), the ISCOM matrix preparation was mixed with PBS (pH 7.4) 1:1. For the recombinant protein vaccine (VAC), the ISCOM matrix preparation was mixed 1:1 with the recombinant protein solution. The doses were 2 ml of adjuvant (ADJ) and vaccine (VAC) for both primary and booster vaccinations. The control (CTRL) vaccine was a 2 ml dose of 0.9% saline.

SAM Determination

Color slides were projected onto a standardized ruler template to equalize magnifications for subsequent tracing of corneal ulcers. Three free-hand drawings of each ulcer were made on paper. Tracing images were digitized and corneal ulcer surface areas were determined with NIH-image (version 1.62). The mean ulcer size was determined from the three tracings. The limit of detection was 0.008 cm^2, the area that corresponded to a 1 mm diameter circle. Surface areas less than this were excluded from analysis. Corneal ulcers resulting from scratches were excluded from the analysis. Ulcers resulting from foxtails or suspect foxtails were not included in the analysis on the first week the ulcer was first noted. If eyes remained ulcerated on subsequent weeks, SAM were entered into the analysis.

Data Analysis

The cumulative number of calves with corneal ulcer SAM > 0.008 was determined each week for the 20 week trial period. For this analysis, calves were only counted one time. Chi-square analysis was used to compare differences in the cumulative numbers of calves with corneal ulcers during each week.

Commercial software (Statview and SPSS were used for statistical analyses). Differences were considered significant at $P<0.05$.

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